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Infectious Multiple Drug Resistance in the Enterobacteriaceae

Annual Report

Stanley Falkow, Ph.D.

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A new quantitative assay for the determination of E. coli heat-stable enterotoxin has been developed which is about 10-fold more sensitive than existing methods. A strategy for the development of subunit vaccines has been tested using specific peptides of the binding region of cholera toxin. Although there have been numerous reports of the relationship between E. coli LT toxin (and cholera toxin) and a putative enterotoxin of Salmonella typhimurium, we have been unable to demonstrate any DNA

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sequence homology between known toxin sequences of E. coli LT, or cholera toxin with the nucleic acid of [illegible]

INTRODUCTION

During the past contract year our laboratory has initiated several new aspects of research dealing with the molecular biology of enteric bacterial pathogens and their toxins. In addition, we have continued to extend our earlier findings dealing with the enterotoxins of Escherichia coli, notably the heat-stable enterotoxin. Three studies are summarized in this report. One has been submitted and accepted for publication. The two others are new projects in varying stages of completion.

I. A new quantitative assay for the determination of the E. coli heat-stable enterotoxin.

Enterotoxigenic E. coli causes diarrheal disease by elaborating two different toxins: a high molecular weight, heat-labile enterotoxin (LT) and a low molecular weight, heat-stable enterotoxin (ST). Several convenient and sensitive in vitro assay systems are available for LT which take advantage of the ability of this toxin to activate adenylate cyclase and elevate cAMP levels in a variety of cell culture systems. However, the assay for ST is based on the suckling mouse bioassay. This in vivo assay suffers from the inconvenience of requiring a steady supply and processing of large numbers of suckling mice. Furthermore, the assay is, at best, semiquantitative in that toxin concentrations are estimated by determining the lowest serial dilution of a culture filtrate which yields a positive secretory response in mice. We have worked out a relatively simple in vitro assay for ST based on the observation that guanylate cyclase is activated by this toxin in membranes of intestinal mucosa. Essentially one isolates intestinal cells from rats, and after homogenization and centrifugation, the particulate fraction may be employed for the assay of ST using a guanylate cyclase assay. Cell fractions can be retained in the freezer for at least three months and the assay system requires less than one hour. The assay is convenient, quantitative, about 10-fold more sensitive than the suckling mouse bioassay and can be performed with crude culture filtrates. (The complete details of our findings along with the appropriate literature references are given in considerable detail in J. Infect. Dis. 149:83-89, 1984 (reprint attached).)

Most clinical and basic research laboratories and certainly the military laboratories with which we are familiar still employ the suckling mouse bioassay. We believe that the assay reported here will facilitate rapid screening of samples for the presence and amount of ST and will prove to be cost-effective.

II. New Approaches to Bacterial Vaccines.

Last year we proposed to initiate studies by which we could dissect the fine molecular structure of cholera toxin and thereby create a general strategy by which subunit vaccines might be directly synthesized in the laboratory. These studies not only serve to provide a novel approach to vaccine development but also provide a means to understand better the molecular mode of action of bacterial toxins. We have made reasonable progress on this project during our first year of work and our results are summarized in the following sections.

A. Plan of Study. Cholera toxin is an 86,000 dalton protein composed of the three polypeptide chains A1, A2 and B with molecular weights of 22,000, 5,000 and 11,500, respectively. A disulfide bridge links the A1 and A2 chains to form toxin subunit A (CT-A) which associates non-covalently with a pentameric aggregate of B-chains, subunit B (CT-B). The amino acid sequences of both subunits have recently been determined using recombinant DNA methods and DNA sequencing, some of which was developed in this laboratory under previous contract funding.

The molecular basis for the biological effects of CT has been extensively investigated: toxin liberated in the small bowel lumen by cholera vibrios binds to specific epithelial cell surface receptors. In particular, the B subunit associates with the oligosaccharide moiety of the plasma membrane monosialoganglioside, GM-1. Subsequently, subunit A enters the plasma membrane and catalyzes the ADP-ribosylation of a guanyl nucleotide binding protein, resulting in irreversible activation of adenylate cyclase. The ensuing increase in intracellular levels of cAMP provoke hypersecretion of chloride, bicarbonate and water (1,2).

Although the sequence of physiological events initiated by CT has been elucidated, the chemical basis by which this protein exerts its effects remains undefined. Specifically, the molecular determinants which serve to bind CT to its receptor are largely unknown. Recognizing the importance of this system as a model of receptor-ligand interactions in general, and of carbohydrate-protein binding in particular, we chose this aspect of the molecular definition of CT upon which to concentrate. The specific objectives of this sub-project bear repeating here since they are still relatively novel as an experimental approach. The specific objectives are:

1. To create a structural model of the cholera B subunit using computer-assisted prediction of secondary structure and hydrophilicity. These studies will help define various domains of the CT-B subunit and whether they are within or on the surface of the protein. Antibodies to these domains will be raised and studied in competitive binding assays.

2. To identify amino acid residues which mediate GM-1 receptor recognition. The receptor binding domain will be sought in several ways. First, a "peptide library" will be constructed. Each entry in the library--containing synthetic peptide analogues to interesting regions of the primary structure as well as proteolytic fragments cleaved from the native protein by chemical or enzymatic means--will be examined for receptor binding function. Second, polyclonal and monoclonal antibodies of known specificity will be assessed for ability to block or permit binding of CT-B to GM-1. The amino acids to which a "permissive antibody" binds can be unambiguously assigned a noncritical role in mediating receptor recognition. Third, anti-idiotypic antibodies to anti-GM-1 antibodies will be raised. This immunoglobulin may contain an "internal image" of GM-1 and therefore recognize the receptor binding domain of CT-B itself. Finally, mutant proteins will be "engineered" by oligonucleotide-directed (site-specific) mutagenesis to manifest specific alterations in the charge, hydrophilicity, secondary structure or bulk of critical amino acid residues. The effects of these alterations upon function will be assessed.

B. Results and Discussion

1. Since the primary objective of our proposed research is to locate and characterize the receptor binding domain of CT-B, we began our work by a prediction of the structure of CT-B. The amino acid sequence of CT-B according to the data of Lai (3) as well as from nucleotide sequencing studies was subjected to the hydrophilicity analysis of Hopp and Woods (4). Figure 1 depicts the results of this study. Areas of greatest average hydrophilicity, residues 30-37, 60-71 and 79-84, are predicted to reside in external portions of the protein and to constitute potential antigenic determinants. By contrast, hydrophobic regions, particularly 38-42, 73-78 and 85-90, are predicted to be buried within the macromolecule or to stabilize polymeric structure. Using the predictive rules of Chou and Fasman (5), the relative probabilities of alpha-helix, beta sheet and beta turns were calculated, (Figure 2) and a model of secondary structure proposed (Figure 3).

2. Selection of putative receptor binding domains. The predicted models of CT-B structure presented us with several attractive candidates for the receptor binding domain according to several postulated criteria. First it has been demonstrated (6) that GM-1 and its oligosaccharide moiety induce a 12mm blue shift in the fluorescence emission maximum of cholera toxin and the CT-B subunit--changes which could reflect local flexibility in local structure. Second, critical regions of the protein may contain both hydrophilic and hydrophobic residues--both types of amino acids are important in creating an appropriate microenvironment for the binding of a glycosidic compound. Third, positively charged residues may play a fundamental role by participating in electrostatic binding with the sialic acid moiety of GM-1. Fourth, the active site may contain a guanidinium group since modification of an arginine (7) abolishes binding function without disruption of secondary or quatenary structure.

Two regions of the protein seem especially interesting since they satisfy all of the postulates discussed above. Analogues of these regions, designated synthetic peptide I and synthetic peptide II (SPI and SPII)--corresponding to amino acids 26-39 and 61-76 respectively (Figure 4)--may exhibit important functional properties and therefore constituted good prospects for chemical synthesis. (A computer search of a library of known protein sequences by our associate Stephen Hirschfeld highlighted the potential importance of SPI.) The amino acid sequence 26-39 was found to be strongly homologous with two types of human interferon, glycoprotein hormones, LH, FSH, TSH, and hCG as well as two toxins from snakes (Figure 5). All of these molecules have been demonstrated or postulated to bind to glycoproteins or glycolipids. This preserved sequence suggests the existence of amino acid residues critical for the postulated GM-1 binding site. Furthermore, the conserved region is approximately the same distance from the amino acid terminus in each of these molecules. To begin our structure/function studies of the CT-B subunit, SPI was synthesized and examined.

3. Studies with SPI. The SPI peptide was synthesized by the Merrifield technique (8) and the completed peptide simultaneously deprotected and cleaved from the solid support by hydrolysis with hydrogen fluoride. The authenticity of the resulting peptide was confirmed by amino acid analysis. We examined the potential significance of SPI by two methods: binding assays which assess the capacity of the peptide to bind to GM-1 and blocking

experiments to determine if antibody to SPI can, when bound to the intact subunit, abolish receptor binding function.

The affinity of SPI for GM1 was assessed by direct binding using ^{125}I-labeled peptide and by competitive inhibition using nonlabeled peptide and ^{125}I-labeled CT-B. No detectable affinity could be demonstrated by these tests. Therefore, antisera to the synthetic peptide was elicited in rabbits by systemic immunization. SPI was prepared for immunization by two methods: crosslinkage with gluteraldehyde and coupling to a carrier protein, keyhole limpet hemocyanin. Preimmune sera were collected from New Zealand white rabbits and the immunization protocol was carried out. (We employed 100ug of each conjugate and 100ug of pure peptide in complete Freund's adjuvant (1:1 v/v) administered by multiple intramuscular and subcutaneous injections. Booster inoculations were given in incomplete Freund's adjuvant at monthly intervals and the preimmune and immune sera assessed by an ELISA method.) Antibodies to SPI bound intact CT-B at serum dilutions of 1:8 at three months and 1:64 at four months. Blocking studies, examining the functional significance of this antisera are in progress.

In addition to these studies we have begun studies with a family of proteolytic fragments generated from CT-B and other candidate peptides have been synthesized and are also under study. We have also synthesized conjugated and tritiated oligosaccharide for binding studies. This work is still in its infancy but we maintain a high level of enthusiasm about its eventual success.

III. Studies on the putative enterotoxin of Salmonella typhimurium. We proposed to examine the possible relationship between the reported cholera-like toxin of S. typhimurium and E. coli LT. It seemed that such a relationship might occur since we had found a reproducible hybridization signal between certain cloned fragments of S. typhimurium DNA and the purified DNA sequences encoding E. coli LT. We have pursued these studies. While there is little doubt that the Salmonella chromosome contains DNA sequence which shares some degree of nucleotide sequence homology with a short segment of E. coli LT-specific genetic sequences, we have been unable, despite repeated attempts to isolate or otherwise demonstrate functional toxin biosynthesis by S. typhimurium strains. Dr. J. Mekalanos of Harvard University has similarly found DNA hybridization between Salmonella DNA and cloned cholera toxin genes (personal communication). He, like us, cannot, however, demonstrate any linking of toxin activity nor isolate a functional toxin-specifying gene from Salmonella.

Because of our puzzling, yet negative, results, we have decided to abandon this project. In its stead, we propose to examine the molecular nature of the outer surface components of Salmonella responsible for its adherence to epithelial cells. These clones were discovered during our search for the putative toxin gene. We find that there has been no prior characterization of the pili genes of Salmonella and it seems a good candidate for further study. Complete details of these studies will be provided in our next annual report. In addition to the genes for adherence, we are also screening the gene library of Salmonella for genes which may govern the invasive capacity of these bacteria for epithelial cells. Clearly since we have established a "bank" of Salmonella DNA sequences within E. coli, in theory at least, we should be able to focus upon several aspects of Salmonella pathogenicity.

References Cited

1. Kassis, S., et al. (1982) J. Biol. Chem., 257:1248.

2. Tsuru, S., et al. (1982) J. Gen. Microbiol., 128:497.

3. Lai, C.Y. (1977) J. Biol. Chem., 252:7249.

4. Hopp, T.P., et al. (1981) Proc. Natl. Acad. Sci. USA, 78:3824.

5. Chou, P.Y., et al. (1978) Ann. Rev. Biochem., 47:251.

6. Fishman, P.H., et al., (1977) Fed. Proc. Am. Soc. Biol., 36:669.

7. Duffy, L.K., et al., (1979) Biochem. Biophys. Res. Comm., 91:1005.

8. Merrifield, R.B. (1963) J. Anal. Chem., 85:2149.

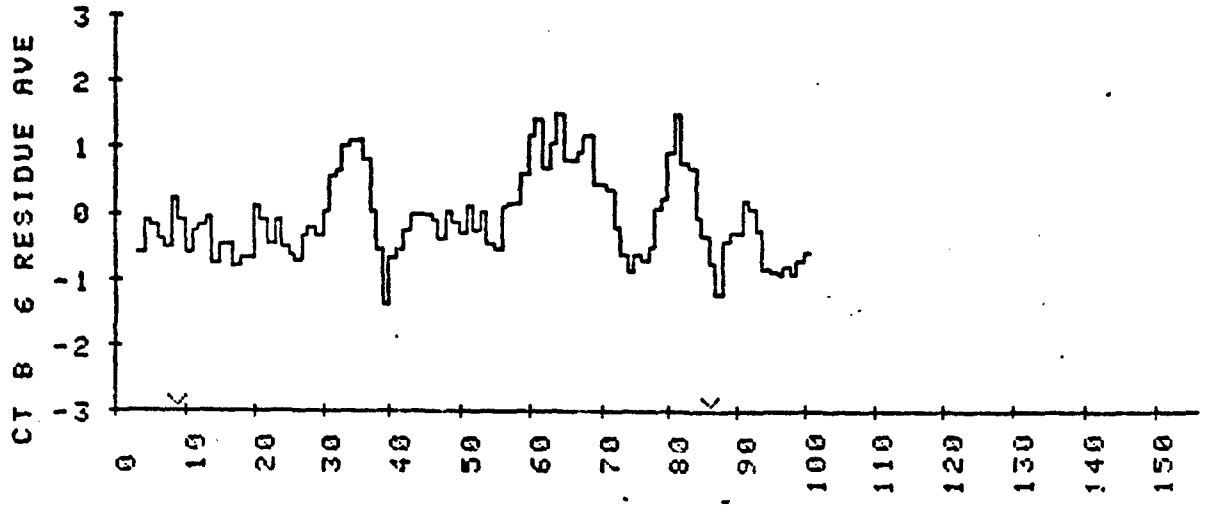


Figure 1. Hydrophilicity profile of the CT B-subunit. Hydrophilicity average (on a scale of +3 to -3.4) at residue x is calculated across 6 residues, from x-3 through and including x+2, according to the method of Hopp and Woods (4). The "v" on the x-axis represents locations of cystine.

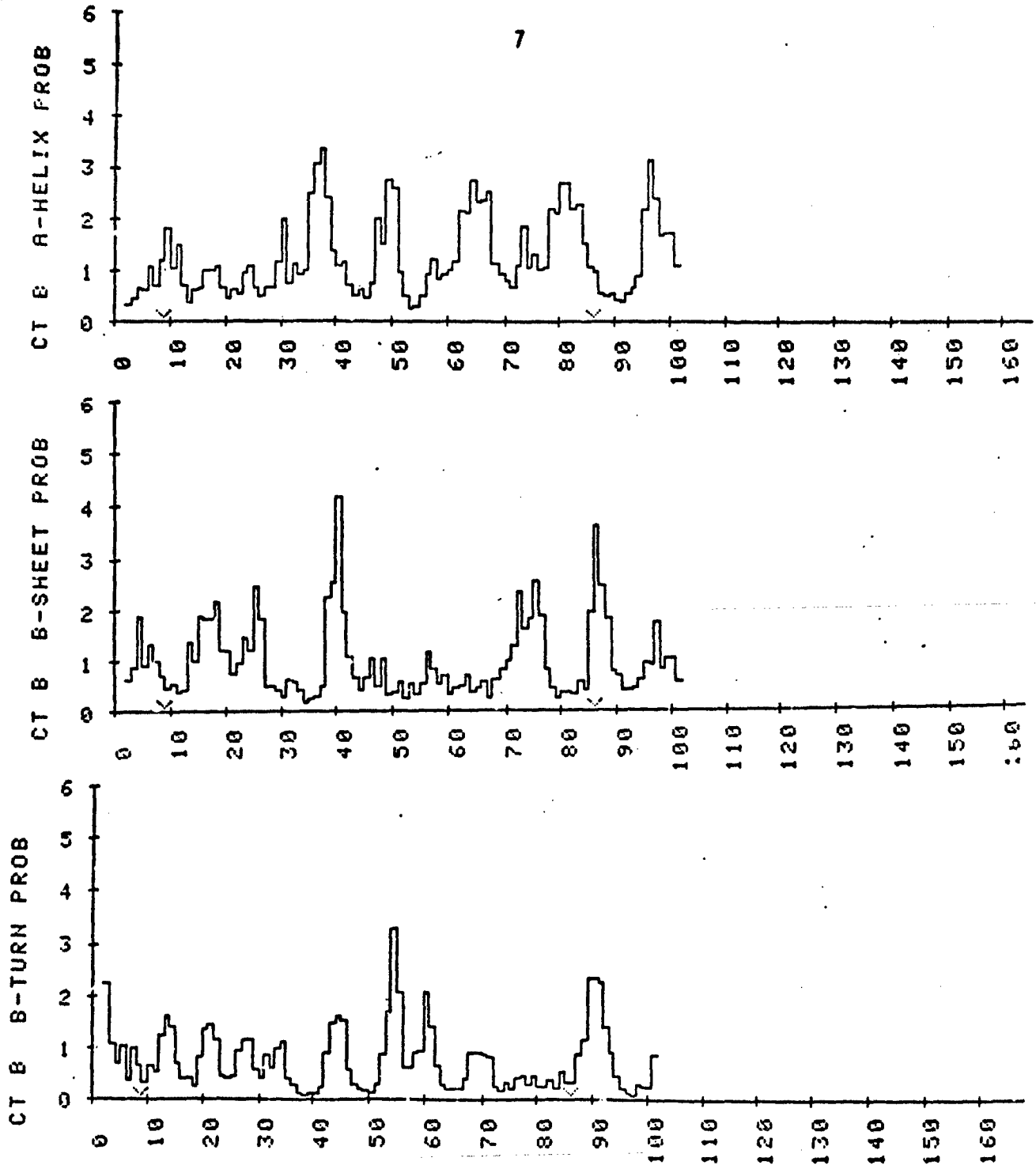


Figure 2. Secondary structure prediction of CT-B. The relative probabilities of alpha helix, beta sheet, and beta turn (6 high to low) are plotted against sequence position, according to the methods of Chou and Fassman (5).

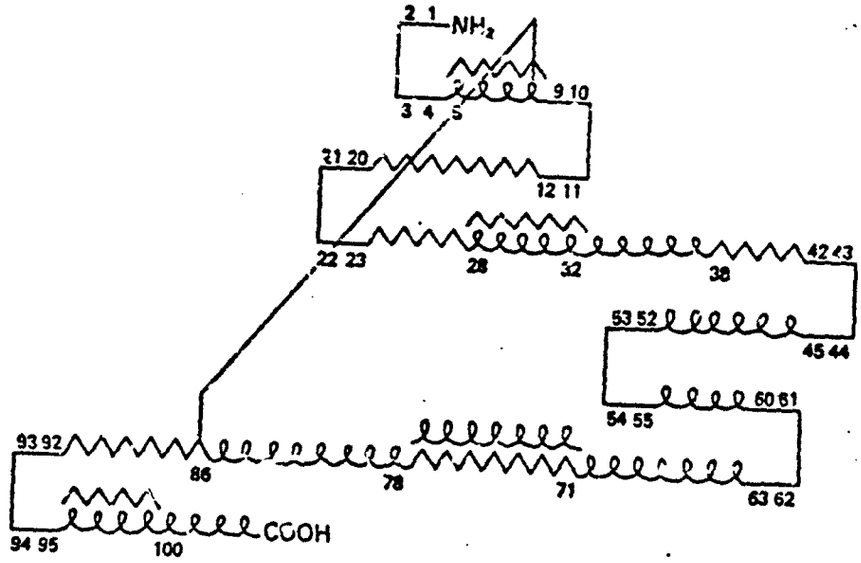
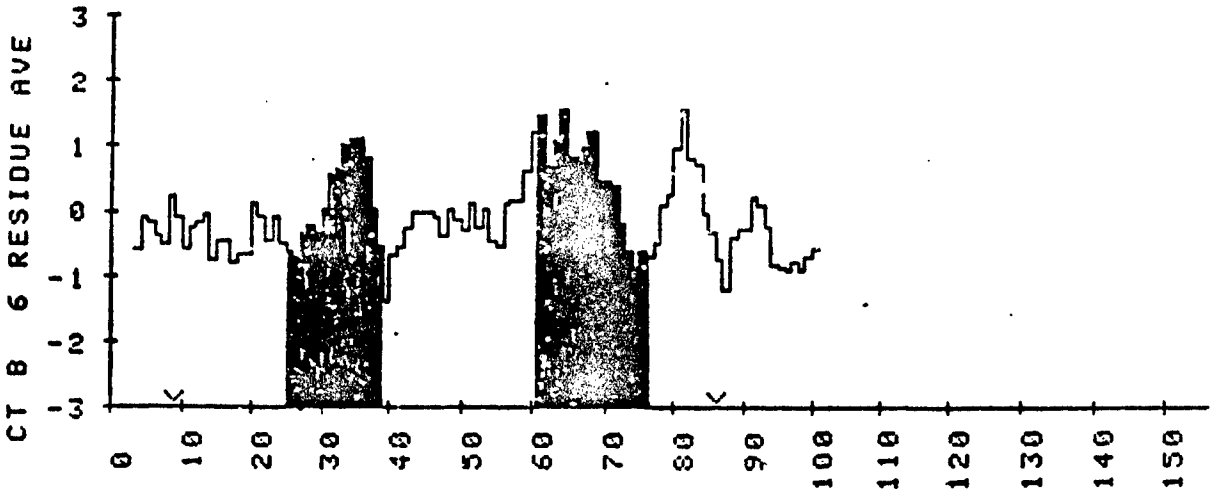


Figure 3. Secondary structure model of CT-B. Each amino acid residue was assigned to either and alpha-helix (), beta sheet (^) or beta turn using the predictive rules of Chou and Fassman (5). Two secondary structures were equally likely in several cases. The position of the intra-chain disulfide bridge is indicated.



Hydrophilicity Profile of the B Subunit

SPI

H_2N - Ser - Tyr - Thr - Glu - Ser - Leu - Ala - Gly - Lys -
26

Arg - Glu - Met - Ala - Ile - COOH
39

SPII

H_2N - Gln - Lys - Lys - Ala - Ile - Glu - Arg - Met - Lys -
61

Asn - Thr - Leu - Arg - Ile - Ala - Tyr - COOH
76

Figure 4. Synthetic peptide analogues. The amino acid sequences chosen for synthesis of SPI and SPII are depicted and their sites within the CT-B structure illustrated.

Residues	Sequence	Peptide
26-39	S Y T E S L A G K R E M A I	Cholera B
26-39	S Y T E S M A G K R E M V I	E. coli LT-B
21-34	P Q T H S L G S R R T L M L	Alpha-2 Interferon-human
27-40	P E T H S L D N R R T L M L	Leukocyte Interferon-human
36-49	A Y P T P L R S K K T M L V	FSH, hcG-human
33-46	A Y P T P L R S K K T M L V	TSH, LH-human
40-53	A Y P T P A R S K K T M L V	LH-sheep, LH, FSH-bovin
34-47	A Y P T P A R S K K T M L V	LH-porcine
26-39	A Y P T P A R S R K T M L V	FSH-horse
40-53	A Y P T P A R S R K T M L V	LH-horse
20-33	S Y T N F I R A V R G R L T	Ricin D, alpha chain-castor bean
9-22	D Y S K Y L D S R R A Q D F	Glucagon-human
23-36	C Y K K W W S D H R G T I I	Short neurotoxin 1-mid asian cobra
22-36	C Y K K Q W S D H R G T I I	Short neurotoxin 1-forest cobra
22-35	C Y K K T W S D H R G T I I	Short neurotoxin 1-Stoke's sea cobra
22-35	C Y K K T W S D H R G T I I	Short neurotoxin 1-Jameson's mamba
23-36	C Y K K Q W S G H R G T I I	Short neurotoxin 3-banded Egyptian cobra
21-34	C Y Q M Y M V S K S T I P V	Cytotoxin 1-forest cobra
21-34	C Y K M Y M V S N K T V P V	Cytotoxin 1-Indian cobra
21-34	C Y K M T M R G A S K V P V	Cytotoxin 2-Mozambique cobra
21-34	C Y K M M L A S K K M V P V	Cytotoxin 4-Mozambique cobra

Figure 5. Sequence homology between CT-B and other polypeptides. Using a library of known protein sequences, sequence homology between CT-B and other proteins were compared by our collaborator, Stephen Hirschfeld. The homologies are found between proteins known to bind carbohydrate and correspond roughly to the synthetic peptide, SPI.

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A Simple, Sensitive, and Specific Assay for the Heat-Stable Enterotoxin of *Escherichia coli*

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A simple assay for the heat-stable enterotoxin (ST) of *Escherichia coli* was developed on the basis of ST activation of guanylate cyclase in membranes from the intestinal mucosa of mice. ST activated guanylate cyclase in mucosal membranes in a linear fashion over a 50-fold range of toxin concentrations with Mg^{++}-guanosine 5'-triphosphate as substrate. Activation of guanylate cyclase was detectable at concentrations of ST that were five- to 10-fold lower than those resulting in increases in the ratio of gut weight to carcass weight of mice. This assay was used to quantify ST in crude and purified samples from culture filtrates of wild-type strains and recombinant strains of *E coli* containing the gene for ST. Activation of guanylate cyclase was specific for ST; purified cholera toxin and *E coli* heat-labile enterotoxin did not activate guanylate cyclase. Thus, this assay for ST is sensitive, specific, and will facilitate rapid analysis of samples for quantification of ST.

Enterotoxigenic *Escherichia coli* causes diarrheal disease by elaborating two different toxins: a high-molecular-weight, heat-labile enterotoxin (LT) and a low-molecular-weight, heat-stable enterotoxin (ST) [1–5]. Several convenient and sensitive assays are available for LT that take advantage of the ability of this toxin to activate adenylate cyclase and elevate levels of cyclic adenosine monophosphate in a variety of cell culture systems [6, 7]. However, the assay for ST is based on the suckling mouse bioassay originally described by Dean et al [8] and modified by Gianella [9]. This assay has the inconvenience of requiring a steady supply and processing of large numbers of suckling mice. Furthermore, the assay is semiquantitative in that toxin concentrations are estimated by determining the lowest serial dilution that yields a positive secretory response in mice [10]. Here we report a simple assay for ST based on the observation that guanylate cyclase is activated by this toxin in membranes of intestinal mucosa [11–13]. This assay is (*1*) convenient, permitting the processing of large numbers of samples quickly; (*2*) quantitative, allowing direct estimation of toxin levels in a sample; and (*3*) about 10-fold more sensitive than the suckling mouse bioassay. Some of these observations have been reported in abstract form [14].

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Please address requests for reprints to Dr S. A. Waldman, Department of Medicine, Stanford University School of Medicine, 3801 Miranda Avenue, Palo Alto, California 94304.

Materials and Methods

Tissue preparation. Male Sprague-Dawley rats (weight, 200–250 g) were killed by decapitation; the jejunum and proximal third of the ileum were removed and rinsed with ice-cold (4 C) 0.25 *M* Tris-HCl (pH 8.0), 1 m*M* EDTA, and 1 m*M* dithiothreitol (sucrose buffer) [13]. Mucosal scrapings, obtained with the edge of a glass slide from one to three rats, were pooled and homogenized in sucrose buffer at 4 C. Homogenates were centrifuged at 105,000 *g* for 60 min to separate supernatant from particulate fractions. Particulate fractions were divided into aliquots and stored at −70 C; each aliquot was frozen and thawed one time only. Under these conditions mucosal membranes retained the ability to respond to ST for at least 12 weeks.

Guanylate cyclase assay. Guanylate cyclase activity was determined in 100-μl reaction mixtures containing 2–200 μg of membrane protein in 50 m*M* Tris-HCl (pH 7.6), 10 m*M* theophylline, 7.5 m*M* creatine phosphate, 20 μg of creatine phosphokinase (160 units/mg), 1 m*M* guanosine 5'-triphosphate (GTP) and 4 m*M* $MgCl_2$ [15]. Assays were initiated by adding Mg^{++}-GTP and then incubated at 37 C for the indicated times in the

presence or absence of various toxin preparations, crude-culture filtrates, or control buffers. Incubations performed in duplicate or triplicate were terminated by the addition of 0.9 ml of cold (4 C) 50 m*M* sodium acetate buffer (pH 4.0) and subsequent heating for 3 min at 95 C. The amount of cyclic GMP (cGMP) formed was determined by RIA [13, 15, 16].

Suckling mouse assay. Newborn Swiss-Webster suckling mice, one to three days old, were randomly divided into groups of three or more [8, 9]. Each mouse was inoculated orally with 10–50 μl of various toxin preparations, crude-culture filtrates, or control buffers. Mice were killed by cervical dislocation 3 hr after inoculation, and the gut, from the stomach to rectum, was removed. The guts and remaining carcasses from each group of three mice were pooled and weighed. The ratio of gut weight to carcass weight (G/C ratio) was calculated.

Toxin and bacterial strains. The preparations of toxins and bacterial strains used in these studies are summarized as follows. Partially purified ST was provided by Dr Richard Greenberg, Washington University, St Louis. This toxin was purified from *E coli* strain C14 isolated from a child in Brazil (the index patient) with epidemic watery diarrheal disease [17]. These preparations were purified according to the method of Alderete and Robertson [10] up to but not including preparative electrophoresis.

Purified ST was provided by Dr D. C. Robertson, University of Kansas, Lawrence. These preparations were purified from *E coli* strain 431 as described previously [10].

ST was also purified from a recombinant strain of *E coli* designated PSLM004. The techniques used to construct this recombinant strain, which produces >10 times more ST compared to wild-type strains, have been described previously [18]. Purification of ST was done according to the method of Staples et al [19] up to but not including the final step of purification on DEAE.

E coli strain 154113 was obtained from a previously described adult patient in Bangladesh who had watery diarrheal disease and who was subsequently identified as an ST producer [18]. *E coli* strains CDC-1 and CDC-2 were obtained from adults in Mexico with epidemic watery diarrheal disease [20].

In some studies, *E coli* strains were heavily inoculated into trypticase soy broth and incubated at 37 C for 2 hr. Crude-culture filtrates were centrifuged at 11,000 *g* for 15 min, and the supernatant was filtered through a sterile glass filter (pore size, 0.45 μm; Diaflo® membrane; Amicon Corp, Lexington, Mass) to remove remaining organisms and debris. This crude-culture filtrate was then concentrated 20-fold and used in experiments as indicated.

Miscellaneous procedures. Protein was determined by a modification [21] of the procedure originally described by Lowry et al [22]. LT was purified as previously described [23]. Cholera toxin was obtained from Sigma Chemical Co, St Louis. All other reagents were as described previously [13, 15, 16].

Results

Figure 1 compares the effects of various concentrations of ST on guanylate cyclase activity in intestinal membranes and the G/C ratio in suckling mice. Guanylate cyclase activity and the G/C ratio increased over a 30-fold range of toxin. Activation of guanylate cyclase was detectable at concentrations of ST about 10-fold lower than those that resulted in increases in the G/C ratio. Also, the concentration of ST producing a half-maximal increase in guanylate cyclase activity was about 10-fold lower than that producing a half-maximal increase in the G/C ratio. Similar observations were obtained with all preparations of ST and mucosal membranes tested.

Activation of guanylate cyclase by ST occurred without a time lag, and the rate of the reaction was linear for about 5 min (figure 2). Subsequently, the rate of the reaction declined and a plateau in basal and ST-activated activity was observed. In contrast, a measurable increase in the G/C ratio due to ST requires 2–3 hr in suckling mice [9]. Activation of guanylate cyclase by maximally stimulated concentrations of ST was observed over a 10-fold range of membrane protein concentration used in the assay (table 1).

The specificity of guanylate cyclase activation by ST was investigated using purified cholera toxin and *E coli* LT. Incubation of these toxins with intestinal membranes resulted in guanylate cyclase activities that were comparable to basal activity—that is, five- to six-fold lower than ST-stimulated activities (data not shown).

The ability to retain biologic activity after heating is an important characteristic of ST [1–5, 9].

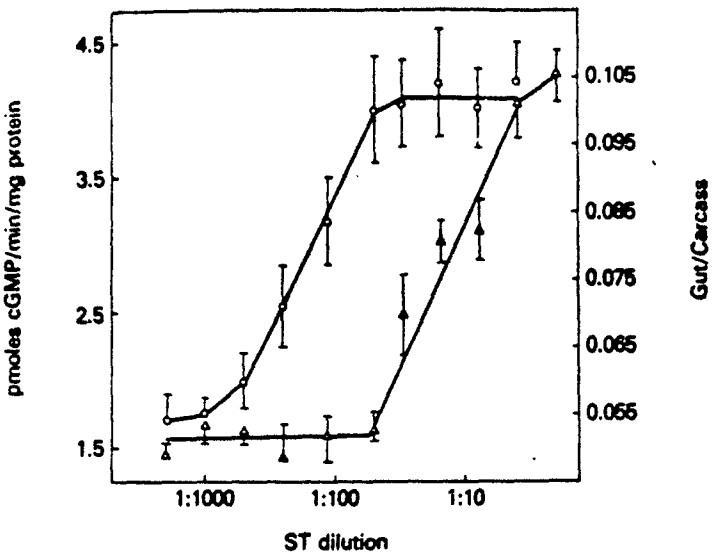

Figure 1. Dose response of guanylate cyclase activation in intestinal membranes or secretion in suckling mice by *E coli* ST. Ten microliters of the appropriate dilution of ST partially purified from *E coli* strain C14 was introduced into assays of guanylate cyclase of intestinal membranes (200 μg of membrane protein per assay) or suckling mice. Assays were performed as indicated in Materials and Methods. Each point represents the mean ± SE of at least three determinations; (O) = guanylate cyclase activity; (Δ) = ratio of gut weight to carcass weight.

We tested the effects of heat on the ability of ST to activate guanylate cyclase in membranes of intestinal mucosa (table 2). Toxins from several strains were incubated at room temperature (about 24 C), 65 C, or 100 C for 15 min and then added to the guanylate cyclase assay. Heat treatment even to 100 C did not alter the ability of toxin from *E coli* strains PSLM004 and C14 to produce maximal activation of guanylate cyclase. However, toxin from *E coli* strain 431 demonstrated a small but statistically significant decrease in its ability to activate guanylate cyclase.

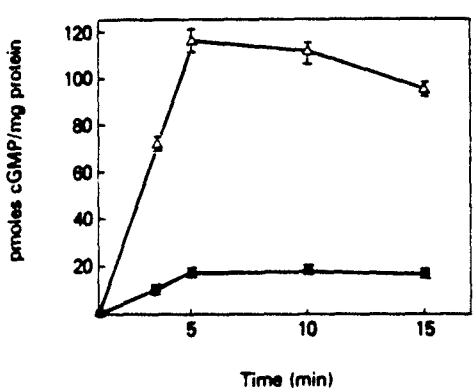

Figure 2. Time course of activation of guanylate cyclase by *E coli* ST. Ten microliters of a 1:20 dilution of ST partially purified from *E coli* strain C14 was introduced into assays of guanylate cyclase. Assays were conducted for the times indicated. Each point represents the mean ± SE of at least three determinations; (Δ) = ST-positive sample; (●) = ST-negative sample.

Once the optimal conditions and specificity of the assay were defined, we used this method to detect and quantify ST in a variety of samples. Quantification of toxin was accomplished by comparing the activation of guanylate cyclase elicited by an unknown sample to that elicited by authentic ST. Thus, a standard curve of the activation of guanylate cyclase by serial dilutions of authentic ST was generated for each experiment (figure 1). One guanylate cyclase unit refers to the amount of ST that elicits a half-maximal activation of enzyme.

The amount of ST was quantified in crude culture filtrates from several strains of *E coli* (table 3). In these experiments, 10 μl of diluted culture filtrate was added to mucosal membranes, and guanylate cyclase activity was determined. Enzyme activity was converted to guanylate cyclase units by

Table 1. Effect of mucosal membrane protein concentration on activation of guanylate cyclase by *E coli* ST.

Membrane protein (μg)	Guanylate cyclase activity	
	Control	ST-activated
165.0	1.26 ± 0.14	4.51 ± 0.12
33.0	1.70 ± 0.19	5.09 ± 0.31
16.5	2.05 ± 0.28	4.49 ± 0.52

NOTE. Data are mean ± SE picomoles of cGMP produced per minute per milligram of protein (four determinations). Maximally activating concentrations of ST (strain C14) were incubated with the indicated amounts of membranes isolated from intestinal mucosa as described in Materials and Methods.

Table 2. Effect of heat on the ability of *E coli* ST to activate guanylate cyclase in membranes of intestinal mucosa.

E coli strain	Temperature (C)	Guanylate cyclase activity*
PSLM004	22	11.07 ± 0.22
	65	11.00 ± 0.10
	100	11.85 ± 0.16
C14	22	9.75 ± 0.13
	65	11.06 ± 0.05
	100	10.44 ± 0.10
431	22	10.27 ± 0.12
	65	9.42 ± 0.18
	100	8.43 ± 0.11

NOTE. Concentrations of ST that maximally stimulated guanylate cyclase were incubated for 15 min at the indicated temperature and then added to enzyme reactions containing mucosal membranes. Basal activities (in the absence of ST) were strain PSLM004, 1.77 ± 0.11; strain C14, 1.19 ± 0.07; strain 431, 0.87 ± 0.03.

* Data are mean ± SE picomoles of cGMP produced per minute per milligram of protein (three determinations).

comparison with a standard curve. ST could not be detected in filtrates from strains producing only LT, which is in close agreement with data presented above and previously [9, 24, 25]. ST could be detected in filtrates of both recombinant and wild-type strains producing ST toxin. Furthermore, the amount of guanylate cyclase activation observed and ST detected corresponded to the dilution of the sample. Thus, the amount of ST (in guanylate cyclase units) detected was appropriately proportional to the amount of sample assayed.

We also used this assay to detect and quantify ST in samples obtained during purification of this toxin (table 4). Purification of ST from culture filtrates of strain PSLM004 was performed as described previously [19] up to and including the first gel filtration step on Sephadex® G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). ST was quantified by comparing the ability of the samples and authentic ST to activate mucosal guanylate cyclase. At each stage of purification, the amount of ST detected was proportional to the amount of sample assayed, in agreement with data presented above. Also, this assay was used to detect the elution position of ST after gel filtration chromatography on Sephadex G-25 (figure 3). The profile of guanylate cyclase activation compares favorably with that of secretion elicited in suckling mice by each fraction. Therefore, this assay is useful for quantifying and detecting ST in samples at various stages of purification.

Discussion

The activation of guanylate cyclase by ST in membranes isolated from intestinal mucosa is well documented [11–13, 26]. This activation is unique since it is highly specific for particulate guanylate cyclase only in intestinal mucosa [11–13, 26]. In contrast, other toxins such as cholera toxin and *E coli* LT activate adenylate cyclase in many cell types [5–7]. Activation of guanylate cyclase by ST has been demonstrated to increase in a dose-dependent manner upon exposure to increasing concentrations of the toxin [12, 13]. These considerations suggested to us that the activation of guanylate cyclase in mucosal membranes might be useful for the detection of ST. The results reported herein indicate that activation of guanylate cyclase in membranes isolated from intestinal mucosa provides a reproducible assay for ST. This assay is about 10-fold more sensitive than the suckling mouse bioassay. To our knowledge, this is the first report directly comparing activation by ST of intestinal secretion in vivo and guanylate cyclase in vitro.

Activation of guanylate cyclase increased in a linear fashion over a 30- to 50-fold range of toxin concentrations, suggesting that this assay could be used to quantify ST directly in samples. This is highly desirable since the current method for

Table 3. Quantification of *E coli* ST in crude-culture filtrates of various *E coli* strains.

E coli strain (enterotoxin)	Dilution	Guanylate cyclase activity*	ST guanylate cyclase units/ml†
None	. . .	0	. . .
CDC-1 (LT)	1:2	0	0 ± 0
	1:4	0	0 ± 0
CDC-2 (LT)	1:2	0	0 ± 0
	1:4	0	. . .
PSLM004 (ST)	1:2	89.4	61.74 ± 5.37
	1:4	32.0	. . .
154113 (ST)	1:2	217.6	119.84 ± 15.98
	1:4	141.3	. . .

* Percentage increase of basal guanylate cyclase activity (1.24 picomoles of cGMP produced per minute per milligram of protein). Data are averages of at least two determinations.

† Data are means ± SE of at least four determinations.

Table 4. Quantification of *E coli* ST during purification of ST from *E coli* strain PSLM004.

Step at which sample was obtained	Dilution	Guanylate cyclase activity*	Total ST guanylate cyclase units†
Crude-culture filtrate	1:5	47.3	23,040 ± 2,520
	1:10	27.1	. . .
Acetone precipitation	1:100	93.8	1,518.8 ± 304.8
	1:200	46.5	. . .
Sephadex G-25 pool	1:100	488.8	619.9 ± 81.4
	1:1,000	200.0	. . .

NOTE. Purification of ST from E coli strain PSLM004 was conducted according to the method of Staples et al [19].

* Percentage increase of basal guanylate cyclase activity (1.29 picomoles of cGMP produced per minute per milligram of protein). Data are averages of at least two determinations.

† Data are means ± SE of at least four determinations.

quantifying ST is by determining the lowest serial dilution of a sample that elicits a positive response in the suckling mouse assay [10, 19]. We introduce here a new standard unit for quantifying ST: one guanylate cyclase unit is the amount of ST that elicits a half-maximal activation of guanylate cyclase in intestinal membranes. Although it is an operational definition similar to the suckling mouse unit [9, 10, 19], the guanylate cyclase unit has the advantage of only needing to be defined once per experiment. Thus, a standard curve of guanylate cyclase activation is constructed with intestinal membranes and authentic ST, allowing for the cyclase unit to be determined. Toxin concentrations in samples are then quantified by relating the activation of guanylate cyclase produced by a measured volume of sample directly to the standard dose-response profile. A typical guanylate cyclase activation profile is shown in figure 1. In this example, guanylate cyclase activation increases in a linear fashion over a 30-fold range of toxin concentration.

Using this assay, we quantified ST in crude-culture filtrates and samples obtained during the purification of that toxin [19]. The activation of guanylate cyclase elicited by a sample was reproducible and appropriately proportional to the dilution of the sample (tables 3 and 4). This was true

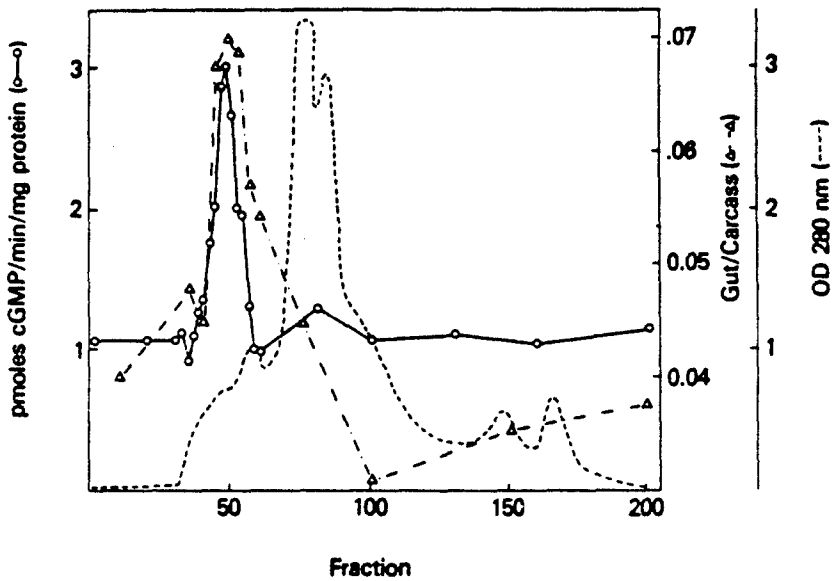

Figure 3. Gel filtration elution profile of partially purified *E coli* ST on Sephadex G-25. ST was purified from crude culture filtrates of *E coli* strain PSLM004 according to the method of Staples et al [19] through the acetone precipitation step. The resulting partially purified material was then loaded on a column of Sephadex G-25 (1.3 × 75 cm), and chromatography was conducted as previously described. Aliquots (10 μl) of 1:10 dilutions of the appropriate fractions were assayed for their ability to activate guanylate cyclase in intestinal membranes or secretion in suckling mice, as described in Materials and Methods. Each point represents the average of at least two determinations; (○) = guanylate cyclase activity; (Δ) = ratio of gut weight to carcass weight; (- - -) = OD units at 280 nm.

for crude culture filtrates, including those produced by *E coli* recombinant strain PSLM004, as well as samples at various stages of toxin purification. Thus, quantification of ST by this method is not limited to purified or partially purified preparations. In table 4, it is interesting to note that ~90% of the total guanylate cyclase units in the crude culture filtrate of *E coli* strain PSLM004 was lost in the initial stages of purification. According to previous reports, ~80% should be recovered with this protocol for purification [19]. However, this protocol is successful in purifying a peptide of 18 amino acids [19] while the recombinant gene encodes a protein of 72 amino acids [18, 27, 28]. These data suggest that this recombinant strain may be producing a form of ST that does not undergo the same posttranslational modification as that produced by wild-type *E coli* strains used by others. Alterations in processing may result in ST molecules having similar physiologic effects but divergent physical characteristics, which result in differences in chromatographic behavior [5, 9–11, 18, 19, 27, 28]. These questions of posttranslational processing of ST are currently being examined with recombinant strains of *E coli* using activation of guanylate cyclase for detection and quantification of the toxin.

In summary, the assay for detection of *E coli* ST reported herein represents a significant improvement over previous methods. This assay is sensitive, with activation of guanylate cyclase detectable at concentrations of ST about 10-fold lower than that required to activate intestinal secretion in suckling mice. Also, this assay is quantitative, with activation of guanylate cyclase increasing linearly over a 30- to 50-fold range of toxin concentrations. In contrast, the suckling mouse bioassay is only semiquantitative [10]. When guanylate cyclase assays are performed in conjunction with an automated RIA system [29], quantification of ST can be achieved in <20 min compared with 3 to 6 hr for the suckling mouse assay. Finally, this assay is convenient, permitting the rapid quantification of toxin in large numbers of samples without the need for maintaining a supply of suckling mice.

References

1. Gyles CL. Heat-labile and heat-stable forms of the enterotoxin from *E coli* strains enteropathogenic for pigs. Ann NY Acad Sci 1971;176:314–22
2. Banwell JG, Sherr H. Effect of bacterial enterotoxins on the gastrointestinal tract. Gastroenterology 1973;65:467–97
3. Sack DA, Merson MH, Wells JG, Sack RB, Morris GK. Diarrhea associated with heat-stable enterotoxin-producing strains of *Escherichia coli*. Lancet 1975;2:239–41
4. Sack RB. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu Rev Microbiol 1975;29: 333–53
5. Giannella RA. Pathogenesis of acute bacterial diarrheal disorders. Annu Rev Med 1981;32:341–57
6. Donta ST, Moon HW, Whipp SC. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science 1974;183:334–6
7. Guerrant RL, Brunton LL, Schnaitman TC, Rebhun LI, Gilman AG. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive, in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect Immun 1974;10:320–7
8. Dean AG, Ching Y-C, Williams RG, Harden LB. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J Infect Dis 1972;125:407–11
9. Giannella RA. Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. Infect Immun 1976;14:95–9
10. Alderete JF, Robertson DC. Purification and chemical characterization of the heat-stable enterotoxin produced by porcine strains of enterotoxigenic *Escherichia coli*. Infect Immun 1978;19:1021–30
11. Hughes JM, Murad F, Chang B, Guerrant RL. The role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*. Nature 1978;271:755–6
12. Field M, Graf LH Jr, Laird WJ, Smith PL. Heat-stable enterotoxin of *Escherichia coli*: in vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. Proc Natl Acad Sci USA 1978;75:2800–4
13. Guerrant RL, Hughes JM, Chang B, Robertson DC, Murad F. Activation of intestinal guanylate cyclase by heat-stable enterotoxin of *Escherichia coli*: studies of tissue specificity, potential receptors, and intermediates. J Infect Dis 1980;142:220–8
14. Waldman SA, O'Hanley PD, Falkow S, Schoolnik GK, Murad F. A simple, sensitive, and quantitative in vitro assay for the detection of heat-stable enterotoxin (ST) produced by *E coli* [abstract]. Clinical Research 1983; 31:50A
15. Kimura H, Murad F. Evidence for two different forms of guanylate cyclase in rat heart. J Biol Chem 1974;249: 6910–6
16. Steiner AL, Parker CW, Kipnis DM. Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. J Biol Chem 1972;247:1106–13
17. Guerrant RL, Moore RA, Kirschenfeld PM, Sande MA. Role of toxigenic and invasive bacteria in acute diarrhea of childhood. N Engl J Med 1975;293:567–73
18. Moseley SL, Hardy JW, Huq MI, Echeverria P, Falkow S. Isolation and nucleotide sequence determination of a

gene encoding a heat-stable enterotoxin of *Escherichia coli*. Infect Immun 1983;39:1167–74
19. Staples SJ, Asher SE, Giannella RA. Purification and characterization of heat-stable enterotoxin produced by a strain of *E coli* pathogenic for man. J Biol Chem 1980; 255:4716–21
20. O'Hanley PD, Cantey JR. Surface structures of *Escherichia coli* that produce diarrhea by a variety of enteropathic mechanisms. Infect Immun 1978;21:874–8
21. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977;83:346–56
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin phenol reagent. J Biol Chem 1951;193:265–75
23. Dorner F. *Escherichia coli* enterotoxin: purification and partial characterization. J Biol Chem 1975;250:8712–9
24. Jacks TM, Wu BJ. Biochemical properties of *Escherichia coli* low molecular weight, heat-stable enterotoxin. Infect Immun 1974;9:342–7
25. Whipp SC, Moon HW, Lyon HC. Heat-stable *Escherichia coli* enterotoxin production in vivo. Infect Immun 1975; 12:240–244
26. Rao MC, Guandolini S, Smith PL, Field M. Mode of action of heat-stable *Escherichia coli* enterotoxin: tissue and subcellular specificities and role of cyclic GMP. Biochim Biophys Acta 1980;632:35–46
27. Lathe R, Hirth P, DeWilde M, Harford N, Lecocq JD. Cell-free synthesis of enterotoxin of *Escherichia coli* from a cloned gene. Nature 1980;284:473–4
28. So M, McCarthy BJ. Nucleotide sequence of bacterial-transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic *Escherichia coli* strains. Proc Natl Acad Sci USA 1980;77:4011–15
29. Brooker G, Terasaki WL, Price MG. Gammaflow: a completely automated radioimmunoassay system. Science 1976;194:270–6